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Cellulose–water interactions during enzymatic hydrolysis as studied by time domain NMR

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Abstract The different states and locations of water within the cellulose matrix can be studied by the use of time domain low field NMR. In this work we show how the state and location of water associated with cellulose in filter paper fibers are affected by enzymatic hydrolysis. Three locations of water were identified in the filter paper; (1) bound water associated with the microfibril surfaces and (2) water in the cell wall or cellulose matrix and (3) capillary water in the lumens and between fibers. The different mechanisms of cellulase enzymes can be seen in their effect on the cellulose–water interactions and the synergistic effects between endo- and exo enzymes can be easily detected by time domain NMR. An interesting observation is that it is possible to link the state and location of water within the cellulose fiber with structural changes upon enzymatic hydrolysis.

Keywords Cellulose · Hydrolysis · Enzymes · Time domain NMR

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Introduction

Enzymatic hydrolysis of cellulose has become an important research area due to the potential use of cellulosic biomass as feedstock for fermentation into ethanol.

The enzymatic breakdown of cellulose to fermentable sugars is done by enzymatic hydrolysis of the glucosidic bonds. The reaction is thus a two-substrate reaction involving both cellulose and water. While there has been considerable interest in the cellulose–enzyme interactions as well as on the cellulose composition, limited attention has been paid to the role of water in the process.

When water is sorbed to cellulose in a plant cell it has properties which are highly different from the properties of bulk water (Kollmann and Côté 1968).

Within the plant cell wall matrix, water is subjected to a number of interactions caused by the chemical and physical composition of the cell wall. Thus the structure and composition of the cell wall produce different states and locations of water, all of which may be important for our understanding of the interactions between cellulose and enzymes.

In the following a general and somewhat simplified description of the state and location of water in lignocellulose is given.

In the range from molecular to micro-scale, the lignocellulosic matrix has several structures that affect the state of water. On a molecular scale the prime source of interaction is the polar groups,

dominated by the hydroxyl groups, which readily form hydrogen bonds with water. At the structural nano-scale level on the surface of the cellulose microfibrils, water is packed in ordered layers or clusters reflecting the crystalline structure of the cellulose. This packed water is here denoted primary bound water, and the density of this water may be as high as 2.5 g/cm^3 (Matthews et al. 2006).

At the cell wall level, sorbed water is located in a porous structure with confined spaces where the water is bound. The mechanisms of bonding are either by capillary forces, by hydrogen bonds to hydroxyl groups on hemicellulose and lignin or by hydrogen bonds to other water molecules already bound to cellulose, hemicellulose and lignin. This type of water is commonly classified as secondary bound water. One also encounters the classification freezing and non-freezing water for the secondary and primary bound water, respectively (Hartley et al. 1992).

Below the fiber saturation point of approximately 25–30% moisture, the major part of the water will be present as primary or secondary bound water within the cell wall Combining the primary and secondary pools of water gives an average density of water in the cell wall of approximately 1.2 g/cm^3. Above the fiber saturation point water fills the cell lumens until full saturation in the area of 60–70% moisture content.

Since water exists in several different states within the cell wall matrix, several issues may be raised in relation to enzymatic hydrolysis.

What is the importance of primary bound water with a density of 2.5 g/cm^3 on top of the cellulose fibrils where the enzymes are active? Do the enzymes affect the primary bound water? Furthermore, the enzyme action may change the state and location of secondary bound water within the cell wall matrix. If so, will it be possible to monitor the effect of enzymatic breakdown on the cell wall matrix structure through the state and location of water?

To answer these questions, time-domain nuclear magnetic resonance (TD-NMR) is a promising technique, in which the relaxation times of the hydrogen nuclei can be used to asses the different states of water in lignocellulose. Two different types of relaxation times can be obtained; spin–lattice (T_1) and spin–spin (T_2). For practical reasons, and because this approach has given useful information, studies of water within solid substrates such as wood, pulp and paper have mostly relied on spin–spin relaxation, and the present work is no exception. The T_2 relaxation time of hydrogen nuclei depends both on how free the hydrogen nuclei is to move, i.e. which molecular environment it is part of and the physical state of that solid or liquid environment. Generally, tight bonding and small compartments shorten the spin–spin relaxation time of hydrogen nuclei.

In some of the earliest work reported on the use of TD-NMR to study cellulose–water interactions, Froix and Nelson (1975), measured both T_1 and T_2 relaxation times for cotton linters in a range of 0–25% moisture content. Four different states of water were identified: Primary bound water on the cellulose crystal, and two types of secondary bound water associated with the cellulose structure as well as bulk water.

Menon et al. (1987) did a comprehensive study on water in wood, finding three pools of water which they assigned to the cell wall, the ray and tracheid lumens and the earlywood tracheid lumens. Also by choosing different tree species, they found that species had an effect on the T_2 values. Species with smaller cell lumens had shorter T_2 values for the lumen water.

Araujo et al. (1993) examined the location of water in white spruce softwood by TD-NMR identifying bound water, lumen water in late wood cells and lumen water in early wood cells. The latter having the largest lumens and thus the longest relaxation time.

Antique paper was examined by Blümich et al. (2003). They assessed paper as a bi-component material made from cellulose and water, and found that T_2 values were correlated to the condition i.e. level of breakdown of the paper.

Elder et al. (2006) used TD-NMR to study water in hardwood chars. They found different distributions between bound and free water as a function of moisture content as well as effects of changing temperatures and pore sizes. In addition, a clear effect of species on T_2 relaxation time as well as an effect of moisture and temperature was observed.

In a study of fungal attack on commercial paper, Capitani et al. (1998) described that by adding a cellulase extract from *Aspergillus niger*, a fast response in the T_2 relaxation times upon addition of the enzymes was found. This response was assigned to changing water pools in what was labeled as amorphous regions of cellulose.

The bulk of the cited work was done at low moisture contents as compared to the conditions that would be applied in a commercial process for conversion of cellulose to fermentable sugars. Such moisture levels will be in the area of 60–80%, thus significantly above the fiber saturation point and presumably with most lumens water-filled.

In the present work we applied TD NMR to examine the states and locations of water in a cellulose–water system subjected to hydrolysis by endo and exo-cellulases as well as a complete cellulase system. Filter paper was chosen as the model substrate as it consists almost of pure cellulose, but still has an intact cellulose matrix and cell wall structure.

Materials

Whatman No. 1 filter paper. Enzymes; purified *Tricoderma longibrachiatum* endoglucanase (EG) EC 3.2.1.4 and cellubiohydrolase (CBHI) EC 3.2.1.91 both from Megazyme, Ireland. Cellulase mixture: commercial product Celluclast 1.5 L from Novozymes A/S, Denmark. The EG and CBH preparations were formulated with 3.2 M ammonium sulphate. The exact formulation of the cellulase mixture is not accessible, but a main component is glycerol.

Experimental

Cellulase treatment

About 0.9 g of filter paper was cut in pieces of approximately 4×4 mm^2. The filter paper was placed in the NMR sample tube and 1.8 ml deionized water with or without enzyme added. The enzyme was mixed with water on a weight basis of 1 mg of protein for each treatment. Mixing of filter paper and water with or without enzyme was done by adsorption only. Temperature during the treatment was identical to the NMR operating temperature 40 °C. The pH of the filter paper–water mixture was 4.9.

HPLC of released sugars

For verification of enzyme activity the content of cellobiose, and glucose was quantified on a Dionex Summit HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80 °C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1}. Samples were filtered through a 0.45 μm filter and diluted with eluent before analysis on the HPLC.

NMR measurement

NMR analyses were done using a Bruker mq20-Minispec, with a 0.47 Tesla permanent magnet (20 MHz proton resonance frequency), operating at 40 °C. The transverse (T_2) relaxation times were determined using the Carr–Purcell–Meiboom–Gill (CPMG) sequence. About 3,000 echoes were collected with a pulse separation of 0.05 ms, the acquisition of 32 scans and a 5 s recycle delay. The magnetization decay curves were analyzed using mono-exponential and bi-exponential fitting routines to determine discreet values for T_2. The Laplace transformation method CONTIN, as described by Provencher (1982) was used to determine relaxation time distributions. This method is only one of a number of different ways to assess CPMG relaxation curves, and one should keep in mind that different models might fit a relaxation curve equally well from a mathematical point of view (Whittall and Mackay 1989). Here, we have chosen to use CONTIN and discrete exponential fitting, and to focus on differences between sample types.

The NMR measurement started 15 min after addition of water with or without enzymes to the filter paper ($t = 0$). Measurements were done at 0, 15, 30, 45, 60, 90, 120 and 360 min. All NMR measurements were repeated on three set of enzyme treatments.

Conditioning of filter paper

For assignment of water pools, samples of filter paper with different moisture contents (5%, 25% and 66%) were prepared. Air dry 5% moisture content was measured as received. Samples at the fiber saturation point (approximately 25% moisture) were prepared by conditioning the filter paper in a dessicator over deionized water for 10 days. Saturated samples (66% moisture) were prepared by adding 2 g of deionized water to 1 g of filter paper. All samples were placed

in NMR tubes and measured according to the description above.

Results and discussion

Prior to assessing the effect of enzymatic hydrolysis, the effect of different moisture levels upon the state and location of water in the filter paper was studied. The fibers in filter paper are derived from kraft pulped softwoods and though they are composed almost purely of cellulose, the overall cell structure (cell wall and lumen) remains intact. In comparison to non-pulped lignocellulosic fibers, however, the filter paper will have a higher porosity and lack of pore-structures due to the breakdown of lignin and pectin components during the pulping process.

The assignments of the observed peaks are based on the observations shown in Fig. 1. All measurements were done in triplicate, and the observed peaks and changes were consistently seen in all measurements. It can be seen that at 5% moisture, water with a short relaxation time of less than 1 ms dominates and only trace amounts of water with longer relaxation times can be seen. At 5% moisture level it is generally recognized that only bound water is present on the cellulose, thus the 1 ms peak is assigned to primary bound water.

At 25% moisture two peaks at 0.7 and 3 ms, respectively, can be seen. This moisture level is just below the fiber saturation point, i.e. no or little water is present in the lumens. Therefore the peak at 3 ms is assigned to less tightly associated secondary bound water situated in the cell wall structure. At the highest level of 66% moisture content, the peaks assigned to bound and cell wall water are clearly visible, but also to be seen is a large peak at 110 ms due to lumen and inter-fiber water bound by capillary forces. While this water pool could be assigned to bulk water, pure water exhibits a T_2 as high as 3 s (results not shown). As a consequence, the 110 ms peak is identified as water bound by capillary forces in the lumen of the cellulose fibers and denoted lumen water. Unbound bulk water as such is not present in the system even at 66% moisture content. Note that the relaxation times for the cell wall and lumen pools are increased as the water is adsorbed and swells the cellulose structure.

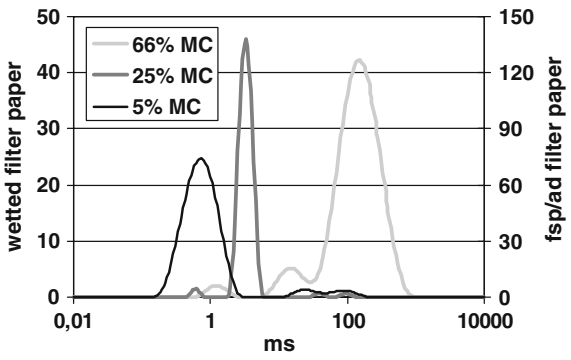

Fig. 1 Comparison of water pools in filter paper found by time domain NMR at three moisture contents (MC). 5% air dried (ad), 25% fiber saturation point (fsp) and 66% wetted. The signal strength is proportional to the water content and for clarity different vertical scales are used

Our assignments of water to three different locations are different to the assignment done by Araujo et al. (1993) on water in white spruce. They state that only bound- and lumen water can be seen, attributing peaks around 10 and 100 ms to water in differently sized cell lumens.

In this work there is a reasonably good agreement with the relaxation times found by Araujo et al. However, we show that up to the fiber saturation point where no or little lumen water is present, there are two distinct peaks; one is bound water and the other around 3 ms must be from water in the porous cell wall. When the moisture content is increased to 66%, the cell wall swells and the relaxation times increase, but we still observe a distinct intermediate peak between the primary bound water and lumen water. Similar observations at different moisture levels on early- and late wood cells from softwoods confirm our assignments. The distinction between primary bound water, cell wall secondary bound water and lumen water in plant cells detected by TD-NMR, therefore appears to be generic (Thygesen et al. under preparation).

We therefore conclude that three different pools and two different states of water can be seen in the filter paper at 66% moisture content; primary bound water tightly associated with the cellulose fibrils, cell wall water—secondary bound by capillary forces or hydrogen bonds in the cell wall, and lumen water—secondary bound capillary water in the cell lumen or between fibers.

With these assignments in mind, we now turn to the measurements on filter paper. For the control samples (water only) it can be seen that the fiber

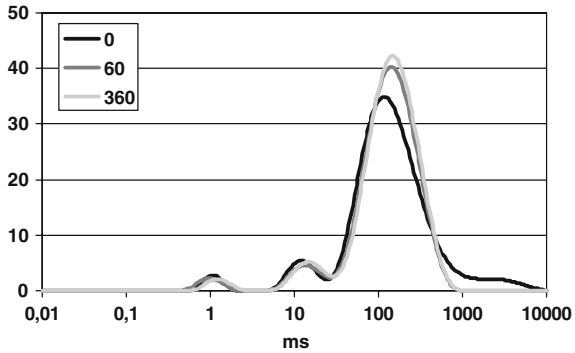

Fig. 2 Control treatment deionised water only. Time domain NMR recorded at 0, 60 and 360 min. For clarity only the measurements at 0, 60 and 360 min are shown

lumen water peak becomes narrower with time as the cellulose adsorbs the water. This is interpreted as an increase in porosity, when the sorbed water swells the cellulose structure and increases the capillary bonding of the lumen water, see Fig. 2.

To ensure the enzymes where active, glucose production was checked by HPLC confirming an increase in glucose throughout the test period. For all three enzymes 0.4–0.7% of the cellulose was hydrolyzed to glucose or cellobiose. At such a low level of enzymatic breakdown it can be assumed that the fiber structure is fully intact. For all enzymes tested, controls with heat inactivated enzyme were performed. The inactivated controls containing the full enzyme preparations were identical to the water only control (results not shown), and we conclude that under the applied conditions, there is no effect of the enzyme formulations or the protein itself on the T_2 values.

Upon the addition of endoglucanase to the filter paper, the T_2 distributions are changed compared to those of the controls (Fig. 3a). The main effect can be seen on the T_2 for the lumen water which when displayed on a linear scale (Fig. 3b) has a more narrow distribution i.e. a stronger adsorption of capillary bound water in the lumen region. This observation can be explained by the mechanism of the enzyme, randomly cleaving the cellulose chains inside the fibrils. This introduces cavities and micropores inside the cellulose structure, increasing the ability of the water to interact with the cellulose as seen on Fig. 3a. This interpretation is also confirmed by observations of Dourado et al. (1999) who found that cellulase treatment of cellulose increased the water holding capacity.

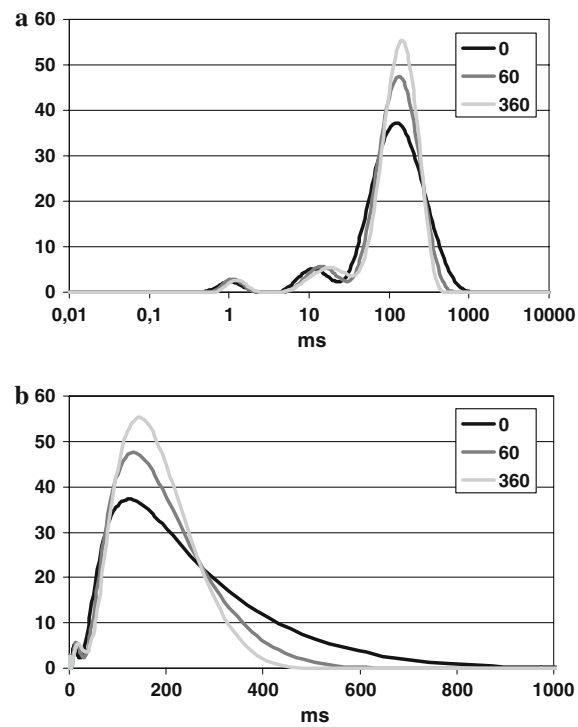

Fig. 3 Time domain NMR spectra of endoglucanase treated filter paper from 0 to 360 min. (**a**) logarithmic scale showing all three water pools; primary bound water, cell wall water and lumen water. (**b**) Linear scale showing how the lumen water is more strongly adsorbed as the enzyme reaction proceeds

It can also be seen how the EG increases the relaxation time of the water associated with the cell wall (Fig. 4). The longer relaxation time can be interpreted as a loosening or opening up of the structure at the earliest point of cellulose breakdown. Thus, the initial action of the EG not only splits the cellulose chains, but may also introduce water into the cellulose structure by the formation of cavities and micro pores.

Contrary to the EG, the cellobiohydrolase (CBH) has no detectable effects on the water pools under the applied conditions. Both the T_2 distributions and the relaxation times found from the peak values are identical to the control (Fig. 5). The activity of the enzyme was confirmed by the release of glucose, but the exo mechanism which cleaves the cellulose from the ends does not cause any structural changes that would affect the state or location of the water.

The final enzyme tested was the commercial Celluclast 1.5 L enzyme preparation from Novozymes. This enzyme mixture is derived from

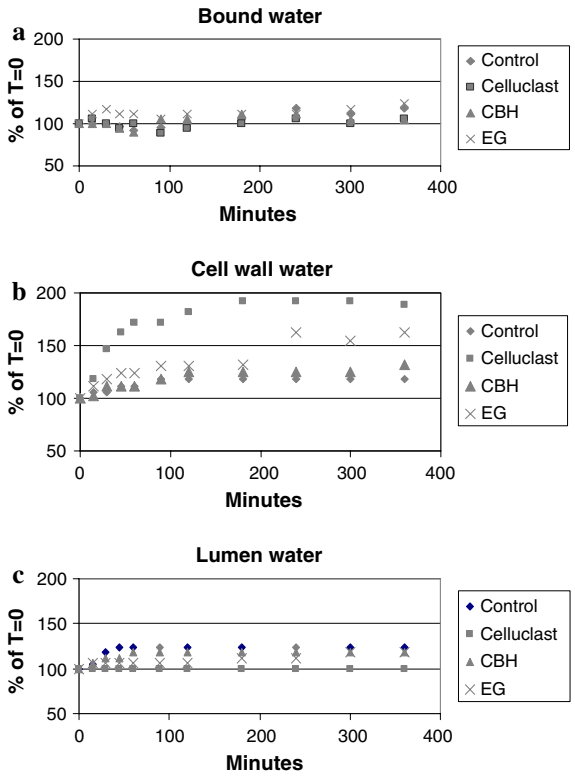

Fig. 4 Development in relaxation times for the different treatments as found for primary bound-, cell wall- and lumen water. The values reported are average for three independent measurements. The vertical scale is the relative change of the T_2 value compared to the relaxation time at $t = 0$

Trichoderma reesei and contains a number of endo- and exo-acting cellulases. Celluclast does not have any significant beta-glucosidase activity, which may cause product inhibition from a build up of cellobiose. However, this was not considered to be relevant for the current study since only the initial phase of the cellulose breakdown was the focus of the work.

The addition of Celluclast has by far the most pronounced effect on the state and location of water (Fig. 6a). The lumen water peak is even narrower compared to the endoglucanase from *T. longibrachiatum*, the cell wall water peak is apparently split into two peaks, and a magnified view (Fig. 6b) of the primary bound water peak shows that it is also narrowed, similar to the lumen water peak. The reason for the change in the primary bound water peak is not known.

The increased relaxation time for the main part of the cell wall water associated with the 25 ms peak indicates a significant loosening or fragmentation of the whole cell wall matrix. Interestingly, this is only associated with a limited release of glucose and cellobiose, and what we observe may be described as enzymatic "drilling" as proposed by Dourado et al. (1999). Thus enzymatic drilling is associated with a

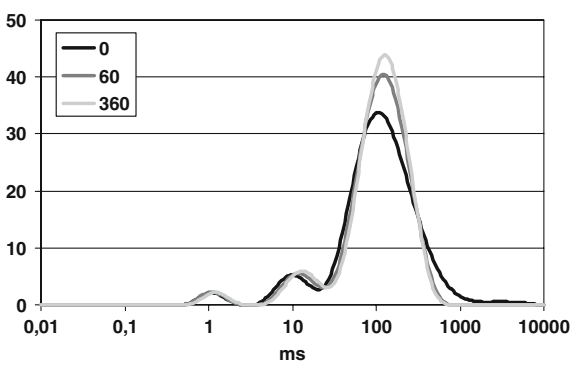

Fig. 5 Time domain NMR spectra of cellobiohydrolase treated filter paper from 0 to 360 min. The distribution and character of water pools are identical to control

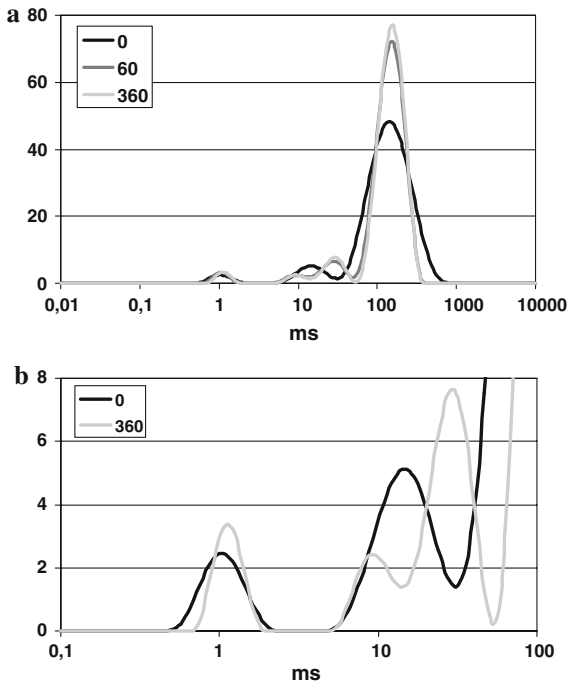

Fig. 6 Time domain NMR spectra of Celluclast 1.5 L treated filter paper from 0 to 360 min. (**a**) Both lumen and matrix water are affected by the cellulase system. (**b**) Expanded view of the cell wall water showing how two different pools of cell water develops

loosening of the cellulose matrix structure creating cavities and micropores, but still maintaining the overall structural characteristics. The remaining cell wall water peak at 9 ms can be interpreted as belonging to non-accessible or recalcitrant structures in the cell wall structure, however, more studies on the time development is necessary in order to elucidate this.

Based on the time dependant development of the cellulose–water interactions and the lack of effect using heat inactivated enzyme preparations i.e. containing both enzymes and stabilizers, we have assigned the observations to the catalytic effect of the enzyme. However, it must be considered whether the changes in the cellulose–water interactions can be caused by the adsorption of cellulose binding domains (CBD) onto the cellulose structure rather than the hydrolytic breakdown. Both the EG and CBH are isolated from *Tricoderma longibrachiatum* and both enzymes have catalytic cores with an CBD attached. The fact that the EG has an time dependant effect upon the cellulose–water interaction and the CBH has none, excludes major effects from the adsorption of the CBD's. The changes in the cellulose–water interactions must be assigned to the catalytic activity of the enzymes. This does not rule out a possible role of CBD's and accessory proteins in the interactions of cellulose and water, but the interpretation of the results in this work should be assigned to the enzymatic hydrolysis.

Considering the development of the relaxation times from 0 to 360 min for all three enzyme preparations (Fig. 4), little or no effect can be seen on the primary bound water. Most likely, higher enzyme protein loadings than the applied approximately 0.1% are required to reveal possible effects on primary bound water. For cell wall water, a clear effect of increasing relaxation times i.e. a degradation and thereby looser cellulose matrix structure can be seen both for Celluclast 1.5 L and EG.

The lumen water shows similar behavior for the control, EG and CBH with slightly increasing relaxation times caused by the swelling of the cellulose cell wall structure, whereas Celluclast 1.5 L stays constant. The latter can be explained by that even though Celluclast 1.5 L causes the most pronounced changes on the cellulose matrix and the cellulose–water interactions, it also increases the porosity and water bonding capacity of the cell wall, which counteracts the effect of swelling and loosening of the cell wall.

The relaxation time behavior found in this work are different from that reported by Capitani et al. (1998), who reported a shortening of T_2 relaxation times upon cellulase addition. Shorter relaxation time does not appear logical, as the breakdown of cellulose should result in a less organized structure and thus longer relaxation times. This discrepancy to the present work is most likely caused by the fact that Capitani et al. (1998) used commercial office paper with a high content of clays such as kaolin.

The results from this TD-NMR study of water during enzymatic hydrolysis are interpreted in terms of its effect on the cellulose matrix structure as illustrated in Fig. 7. What is surprising to the authors are not the observed mechanisms or structures, but the fact that under the applied conditions with no stirring and a relatively low enzyme dosage, the combined action of an endo- and exo-glucanase system caused substantial changes in the cell wall matrix, as observed on the cellulose–water interactions. These changes most likely occur at the molecular level and are at best only marginally detectable by chromatographic or microscopic methods, but their effect on cellulose structure and the cellulose–water interactions are clearly seen by time TD-NMR. The term "enzymatic drilling" is thus a good description of the initial cellulase action, and we believe that it is of prime importance for the overall performance of industrial enzyme preparations for cellulose hydrolysis.

In this work we have used filter paper for simplicity. This substrate is of course not identical to the lignocellulosic substrates to be used in e.g. a commercial cellulose to ethanol process. However, our previous experience on thermally pretreated

Fig. 7 Illustration of the enzyme action upon the structure of the cellulose matrix for endoglucanase (EG), cellubiohydrolase (CBH) and the cellulase mixture Celluclast 1.5 L

wheat straw (Kristensen et al. 2006; Jorgensen et al. 2007), show that the basic factors regulating the enzyme hydrolysis are quite identical to a pure cellulose substrate. The results presented in this work may with some caution be extrapolated to lignocellulosic substrates as well.

Conclusions

The results show that TD NMR can provide detailed information on cellulose–water interactions during enzymatic hydrolysis. During the initial enzymatic hydrolysis of cellulose, the action of the enzyme system is a breakdown and loosening of the cellulose introducing more water into the structure and providing better access for the enzymes. In particular, the cell wall matrix is affected by a combined cellulase system, even under conditions where no stirring is applied. The use of TD-NMR is a promising technique for further elucidation and understanding of the enzyme–cellulose–water system and its interactions.

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